Environmental and Experimental Botany 66 (2009) 389–401

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Rock-degrading endophytic bacteria in cacti

M. Esther Puente[a], Ching Y. Li[b,1], Yoav Bashan[a,c,*]

[a] Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Mar Bermejo 195, Colonia Playa Palo de Santa Rita, La Paz, B.C.S. 23090, Mexico
[b] USDA Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, 3200 SW Jefferson Way, Corvallis, OR 97331, USA
[c] Department of Soil, Water and Environmental Science, 429 Shantz building # 38, The University of Arizona, Tucson, AZ 85721, USA

ARTICLE INFO

Article history:
Received 20 August 2008
Received in revised form 3 February 2009
Accepted 21 April 2009

Keywords:
Bacillus
Cactus
Cardon
Desert
Rock degradation
Nitrogen-fixation
Pachycereus
Phosphate solubilization
Rock weathering
Soil formation

ABSTRACT

A plant–bacterium association of the cardon cactus (*Pachycereus pringlei*) and endophytic bacteria promotes establishment of seedlings and growth on igneous rocks without soil. These bacteria weather several rock types and minerals, unbind significant amounts of useful minerals for plants from the rocks, fix *in vitro* N_2, produce volatile and non-volatile organic acids, and reduce rock particle size to form mineral soil. This study revealed the presence of large populations of culturable endophytic bacteria inside the seeds extracted from wild plants, from seeds extracted from the guano of bats feeding on cactus fruit, in seedlings growing from these seeds, in the pulp of fruit, and in small, mature wild plants, and are comparable in size to populations of endophytic populations in some agricultural crops. The dominant culturable endophytes were isolates of the genera *Bacillus* spp., *Klebsiella* spp., *Staphylococcus* spp., and *Pseudomonas* spp. Based on partial sequencing of the 16s rRNA gene, the isolated strains had low similarity to known strains in these genera. However, these strains have higher molecular similarity among endophytes obtained from seeds, endophytes from roots, and some bacterial strains from the rhizoplane. Seedlings developed from seeds with endophytes contain the similar species of endophytes in their shoots, possibly derived from the seeds. This study shows the involvement of endophytic bacteria in rock weathering by cacti in a hot, subtropical desert and their possible contribution to primary colonization of barren rock. This study proposes that cacti capable of acquiring diverse populations of endophytes may give them an evolutionary advantage to gain a foothold on highly uncompromising terrain.

1. Introduction

The weathering of rock to create new soil is a complex interaction of physical weathering, chemical reaction from air pollutants, soil moisture, acid rain, and biological processes (Hirsch et al., 1995a,b; Goudie and Parker, 1999). Microorganisms covering surfaces, fissures, and pore spaces of rocks sometimes form biofilms (De la Torre et al., 1993; Gorbushina et al., 2002) that contribute to the breakdown of rock. Microorganisms involved in rock weathering are lichens (Barker and Banfield, 1998), fungi (Hirsch et al., 1995b), cyanobacteria (Ferris and Lowson, 1997), many species of bacteria (Adams et al., 1992), and microalgae (Hirsch et al., 1995b). Microbial rock weathering is common in all climate zones, usually acts very slowly (Sun and Friedmann, 1999), and has been observed in hot (Adams et al., 1992) and cold deserts (Friedmann and Kibler, 1980). Little is known about weathering mechanisms, except that some microorganisms produce acids in culture (Hirsch et al., 1995b). Organic acids have also been detected in weathering stones, making this a likely mechanism (Palmer et al., 1991).

Additionally, iron and sulfides in rocks can be oxidized by bacteria and be transformed into new minerals at great sea depths and in deserts (Bach and Edwards, 2003; Bawden et al., 2003; Edwards et al., 2003; Hossner and Doolittle, 2003). Acids produced by microorganisms, as by-products of their metabolism, can dissolve rocks and the resulting minerals benefit microbes and plants (Hinsinger and Gilkes, 1993, 1995; Illmer and Schinner, 1995; Illmer et al., 1995; Chang and Li, 1998; Vazquez et al., 2000; Yamanaka et al., 2003). However, precise data on weathering rates by biological agents in most environments is scarce.

We previously described several species of desert plants, mainly cacti, growing without soil and noticeably weathering rocky cliffs, large rocks, and ancient lava flows in hot desert areas of Baja California, Mexico (Bashan et al., 2002, 2006) and that their rhizoplane population is capable of dissolving minerals and assisting plant growth (Puente et al., 2004a,b).

This study explored endophytic bacteria residing in seeds and tissues of cacti growing in a hot desert and recorded this activity. We hypothesized that some of the seed endophytes moved to the

* Corresponding author at: Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Mar Bermejo 195, Colonia Playa Palo de Santa Rita, La Paz, B.C.S. 23090, Mexico. Tel.: +52 612 123 8484x3668
fax: +52 612 125 4710.
E-mail address: bashan@cals.arizona.edu (Y. Bashan).
[1] Retired.

0098-8472/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.envexpbot.2009.04.010

rhizoplane and participated in rock weathering and transformation of minerals. As a result of this plant–bacteria association, soil formation is accelerated.

2. Materials and methods

2.1. Sampling area, sampling techniques, size, and design

The geographical sources of plants were detailed previously (Bashan et al., 2002). Briefly, plants were taken from volcanic areas in the central Baja California Sur mountain range (Sierra de La Gigante) southwest of the town of Loreto (25–26°N). Relatively young plants of the giant cardon cactus (*Pachycereus pringlei* [S. Watson] Britton and Rose), 5–20 cm tall, growing within rocks, and lacking any direct connection to a soil deposit were sampled. Usually, rock engulfing a plant was carefully broken open with a hammer and chisel to expose the cavity in which the plant was growing, and the entire plant, including its root system were extracted manually. The shoots were discarded and the roots were handled as described later. Two or three young plants were extracted from several rock sites during each of two sampling periods, one during the "dry" season (March 1999, no precipitation since October 1998) and one in the "rainy" season (September 1999; 14.4 mm rain per year). Seeds from a population of cardon were collected at a site near the junction of San Jose and El Rosario Roads (26°25′N, 112°8′W) and from a site at La Purisima–San Isidro (26°36′N, 112°6′W). Seeds were also collected from the following sites: Junction of the local roads Los Planes–El Sargento (24°3′N, 110°6′W), near the village of El Sargento (24°3′N, 110°6′W), the road to El Rosario (23°23′N, 110°20′W), near the village of El Triunfo (23°49′N, 110°9′W), near the village of San Pedro (23°29′N, 110°12′W), junction of the major highways from Todos Santos–San José del Cabo (23°25′N, 110°13′W), and the El Comitán federal preserve (17 km west of the city of La Paz). Bat feces in the El Comitán Federal Preserve were collected for extracting seeds.

2.2. Microscopic observations, light and fluorescent microscopy, and field emission scanning electron microscopy (FESEM)

Histological studies were carried out on 10-day-old cardon plantlets grown from disinfected seeds (described later). The roots and stem were fixed in FAA solution (50% ethyl alcohol:5% glacial acetic acid:10% formaldehyde, v:v:v) by applying intermittent vacuum. Afterwards, the roots were cut into 4 mm segments and dehydrated in increasing ethanol series (50%, 70%, and 95%, and two final changes at 100%). The fixed and dehydrated samples were imbedded in glycol methacrylate plastic (Feder and O'Brien, 1968), and cut (5–8 μm thick cross-sections) using a rotary microtome, 5 mm above the root emergence area. The specimens were mounted on glass slides, stained with 0.05% toluidine blue in 0.02 M sodium benzoate buffer, pH 4.4 (Strzelczyk and Li, 2000), and examined under a differential interference contrast (DIC) microscope (Olympus American, San Diego, CA). Total bacteria (living and dead, culturable and non-culturable) in the seeds were made visually distinct with the fluorescein isothiocyanate (FITC) stain method (Babiuk and Paul, 1970) and for the number of viable bacteria in the seeds by the fluorescein diacetate (FDA) stain method (Ingham and Klein, 1984). The slides were observed and counted under an episcopic fluorescent microscope (Leitz Laborlux-S, Wetzlar, Germany).

For scanning electron microscopy, root samples were initially kept for 6 h in an icebox during transport to the laboratory, and then overnight at 4 ± 1 °C. Root samples, 0.5–1.5 cm long, were then fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and sliced with a sterile razor in half after fixation. The following day, roots were rinsed in the same buffer, and dehydrated in a series of ethanol concentrations increasing from 30% to 100% for 20 min each, and finally with isoamylacetate. After dehydration, samples were dried with CO_2 in a critical point dryer (Samdri-PVT-3B, Tousimis Research, Rockville, MD).

An alternative technique to observe endophytic bacteria within inner root segments was also used, in which pieces of roots were flash frozen in liquid nitrogen. While still submerged in liquid nitrogen, the roots were cut and the epidermal cell wall was "peeled". Then, the roots were transferred to a vacuum evaporator for drying. The dried samples were fixed to stubs with double-sided adhesive tape and coated with 30 nm 60:40%, gold-palladium alloy foil in a sputter coater (Edwards S150B) and then examined at 7 kV with a field emission scanning electron microscope (FESEM; AmRay 3300FE, Advanced Metals Research, Bedford, MA). Endophytes in fruit were detected in 1 mm thick pulp samples after preparation. The pulp samples were immediately frozen in liquid nitrogen, then exposed to high vacuum (50–100 mTorr vacuum) for 15–20 min, then coated with palladium, and examined at 15 kV with a scanning electronic microscope (Hitachi model S-3000N, Japan).

2.3. Bacterial isolation, identification and enumeration from seeds and plants

To isolate culturable root endophytic bacteria that are capable of rock weathering, root fragments (about 1 cm long) were extracted from rock cavities. Adhering rock particles were separated from roots by washing with sterile water. Roots were then immersed in a solution of 2% Tween-20 for 10 min with mild rotary agitation, and then thoroughly washed with sterile water. The root surfaces were then sterilized in 1.5% (v/v) 4% sodium hypochlorite solution (Aldrich) with agitation for 5 min at ambient temperature (27 ± 2 °C) and thoroughly rinsed with sterile distilled water in a laminar flow hood. Small pieces of root tissue (0.1–10 mm) that had been removed from either the side or the center of the vascular cylinder were placed on top of solid, Rennie's N-free medium (Rennie, 1981) in immuno-flasks and incubated for 10 days at 30 ± 1 °C. This is one of the most commonly used procedures to isolate nitrogen-fixing bacteria. Additionally, the root was then cut longitudinally with a sterile razor and incubated on Rennie's N-free medium, as described above. Selection criteria for isolation were: capacity to fix atmospheric nitrogen, phosphate solubilization, and dissolving of five rock types, all under *in vitro* conditions. Small pieces of root tissue were removed from the vascular area of the roots and transferred into flasks containing 25 ml Rennie's semi-solid (3 g l^{-1} agar) N-free medium (Rennie, 1981) and incubated for 6 days at 30 ± 1 °C without movement. Serial dilutions of developing microbial suspension were subsequently prepared in 0.06 M phosphate buffered saline (PBS) at pH 7.0 and plated (100 μl $plate^{-1}$) on Henderson's medium (Henderson and Duff, 1963). Each plate contained rock particles (90 μm diameter) of one of the following commercial rock powders at 0.25% w/v: limestone, apatite, granite, quartz (Ward's Natural Science Establishment, USA) and basalt (ancient lava flow at La Purisima, Mexico). Basalt was assayed as follows: rocks were submerged in 1 N HCl solution overnight at 28–33 °C to eliminate possible organic matter, rinsed several times with de-ionized water, and dried at 160 °C for 2 h. Rocks were pulverized in a mill (Sprecher and Schun, Industrial Control, Germany) and sieved to obtain 120 μm particles. Plates were incubated for 24–48 h at 30 ± 1 °C and culturable, potential nitrogen-fixing and rock-solubilizing endophytes were isolated and counted. As *in situ* endophytic microbial colonization of root microsites varied greatly, it was impractical to count microbes directly from the FESEM photomicrographs. We used a fluorescent microscopy technique described later. We selected 17 different culturable bacterial strains according to their colony morphology and then identified

Table 1
Endophytic isolates from cardon plants partly identified by the 16s rRNA.

Bacterial isolate	Accession numbers of GenBank	Closest bacterial species listed in the data base (%)
Bacillus sp. ES1	EF123217	92.28 and 91.81; *B. pumilus*
Bacillus sp. ES2	EF123218	96.83, 96.26 and 96.84; *B. subtilis*
Bacillus sp. ES3	EF123219	93.55 and 93.4; *B. pumilus*
Citrobacter sp. RIZO1	EF123220	87.18 and 87.5; *C. freundii*
Bacillus sp. ENDO 3	EF123221	92.44, 92.3 and 92.3 *B. pumilus*
Bacillus sp. ENDO 4	EF123222	93.39 and 93.4; *B. pumilus*
Bacillus sp. ENDO 5	EF123223	92.42 and 92.42; *B. pumilus*
Paenibacillus sp. RIZO1	EF123224	91 and 90.72; *P. chitinolyticus*
Bacillus sp. ES4	EF123225	94.57 and 94.43; *B. subtilis*
Klebsiella sp. SENDO 1	EF123226	88.25 *Klebsiella oxitoca* and 88.29 *Grimontella senegalensis*
Acinetobacter sp. SENDO 1	EF123227	87.96 and 87.96; *Acinetobacter calcoaceticus*
Pseudomonas sp. SENDO 1	EF123228	91.92, 92.8 and 92.81; *P. putida*
Pseudomonas sp. SENDO 2	EF123229	89.16 and 88.99; *P. putida*
Bacillus sp. SENDO 6	EF123230	95.11 and 94.94; *B. pumilus*
Klebsiella sp. SENDO 2	EF123231	93.57 *Klebsiella* sp., 86.28 *Grimontella senegalensis*, 85.01 *Klebsiella oxitoca*
Staphylococcus sp. SENDO 1	EF123232	94.41 *S. gallinarum*
Staphylococcus sp. SENDO 2	EF123233	92.52 and 91.67, *S. gallinarum*

Analysis was done at the first 300 bp (the 5′ end of the 16S rRNA gene) using Gapped BLAST and PSI-BLAST analyses (Altschul et al., 1997).

by 16S rRNA gene sequence analysis. Nucleotide sequences have been deposited in the GenBank nucleotide sequence data base (Table 1). Strains used for detailed evaluation were six isolated seed endophytic bacteria (*Klebsiella* sp. SENDO 1, *Klebsiella* sp. SENDO 2, *Bacillus* sp. SENDO 6, *Staphylococcus* sp. SENDO 2, *Acinetobacter* sp. SENDO 1, *Pseudomonas sp.* SENDO 2), two positive controls, plant growth-promoting bacteria (PGPB) *Pseudomonas putida* R-20 (Meyer and Linderman, 1986), and the PGPB *Azospirillum brasilense* Cd, ATCC 29710.

Additionally, several bacterial consortia were used: a consortium of the six endophytes listed above, a consortium of the endophytes with each of the PGPB, and the consortium of endophytes with both PGPB.

2.4. Testing endophytic bacteria

2.4.1. Acetylene reduction assay

Isolates of endophytic bacteria taken from the interior of disinfected roots, as described above, were evaluated for N_2-fixation. Five pieces of roots were put into a flask containing Rennie's N-free, semisolid medium (Rennie, 1981), and incubated for 6 days at $30 \pm 1\,^{\circ}C$ without movement. The bacterial pellicle that formed in the growth medium was extracted, serially diluted in PBS and plated on the same medium, solidified with 2% agar, and incubated for 48 h at $30 \pm 1\,^{\circ}C$. Different bacterial colony morphotypes developed and were purified in a conventional manner on the same solid, N-free medium. Since the medium lacked nitrogen, all bacteria were assumed to be N_2-fixing because they grew 4–5 times on medium lacking nitrogen. To verify N_2-fixing activity of the specific bacterial isolates, 10% acetylene was injected into flasks showing bacterial growth. Acetylene reduction assay was determined by gas chromatography (Holguin et al., 1992).

Bacterial isolates were grown in nutrient broth (Difco, BD Diagnostics Systems, Sparks, MD) at $30 \pm 1\,^{\circ}C$ for 18 h at 120 rpm agitation, and harvested by centrifugation at 10,000 *g* for 20 min. Inocula were washed three times in sterile, distilled water and pellets were suspended in saline solution (0.85% NaCl) to a final concentration of 10^9 CFU ml^{-1}. Flasks containing the following constituents were prepared ($g\,l^{-1}$): mannitol, 5; glucose, 10; sucrose, 5; and pulverized rock, 1.5 in 135 ml de-ionized water. Each flask was inoculated with 15 ml of a bacterial suspension. Flasks were incubated for 28 days at $30 \pm 1\,^{\circ}C$ in a rotary shaker (Incubator shaker series 25; New Brunswick Scientific, Edison, NJ) at 150 rpm. Weekly samples were taken for phosphate solubilization analysis, bacterial count on nutrient agar, and pH.

Weathering was initially observed with bacteria grown on Henderson's medium (Henderson and Duff, 1963) or Pikoskaya's medium (Pikoskaya, 1948) at $30 \pm 1\,^{\circ}C$ supplemented with 2.5 $g\,l^{-1}$ pulverized rock (<120 μm diam.) or insoluble phosphate source on solid medium for three rocks (i) marble at <90 μm diam. (Ward's Natural Science Establishment, Rochester, NY), main ingredients calcite ($CaCO_3$) and dolomite ($CaMg(CO_3)_2$), (ii) limestone at <90 μm diam., main ingredient calcite $CaCO_3$, and (iii) insoluble phosphates with an insolubility range from $AlPO_4 > FePO_4\,2H_2O > Ca_{10}(OH)_2(PO_4)$, (Spectrum Chemicals and Laboratory Products, Gardena, CA). Observations of growth and halo production on solid opaque medium were made every 3 days (measured in mm). Sometimes, no defined halo was observed, but the medium lost opacity. *Bacillus megaterium* (DSM 3228, Germany) and *P. putida* R-20 served as positive controls.

The ability of some strains to dissolve phosphates was quantified according to Vazquez et al. (2000), using the phosphate types listed above as substrates in Henderson's and Pikoskaya's media.

2.4.2. Mineral analysis

After an incubation period (described above), the suspended pulverized rock powder and the bacteria residing in the slurry were removed by centrifugation at 1000 *g* for 10 min, and pellet minerals (P_2O_5, K_2O, Ca^{2+}, Mg^{2+}, Na, Mn^{2+}, Fe_2O_3, Cu^{2+}, Zn^{2+}) were analyzed by EPA Method #3015-microwave digestion (nitric acid) (Kingston, 1994) with an atomic absorption spectrometer (GBC Scientific Equipment, Dandenong, Victoria, Australia). The concentrations of phosphate in pellets were determined according to Jackson (1958). Dry pulverized rock and uninoculated wet pulverized rock incubated under the same conditions served as controls. The minerals were analyzed at the same time as those from culture media. Additionally, weathered rock particles were quantified by measuring diameters and surface areas of particles with an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD) before and after inoculation with bacteria.

2.4.3. Organic acid production and analysis

The isolated bacteria were tested for production of organic acids by the test described above. Flasks were incubated for 14 days at $30 \pm 1\,^{\circ}C$. Volatile and non-volatile organic acids were analyzed by gas chromatography (6000 GC, Varian Instrument Group, Sunnyvale, CA), as described earlier (Puente et al., 2004a), using commercial gluconic, propionic, acetic, lactic, formic, n-butyric, oxalic, malonic, methylmalonic, valeric, isovaleric, succinic, hep-

tanoic, caproic, oxalacetic, isocaproic, fumaric, isobutyric acids as standards (Sigma, St. Louis, MO).

2.4.4. Tolerance to high temperature and NaCl

Sodium chloride tolerance was measured on nutrient agar (Sigma) and tryptic soy agar (Sigma) media in Petri dishes, supplemented with increasing concentrations of NaCl at 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0%. Bacteria were inoculated by streaking the agar surface. Plates were incubated for 24–48 h at 30 ± 1 °C, and bacterial growth was evaluated.

For temperature tolerance, bacteria were similarly grown in Petri dishes containing nutrient agar or tryptic soy agar at 45–55 °C for 24–48 h. During incubation, the agar medium did not dry.

2.5. Verification of endophytic bacterial population in seeds, their isolation and internal transfer from seeds to shoot

Fruit were collected from wild plants at nine sites. Seeds were extracted from these fruit in September 2003 and seeds from long-nose bat feces were collected in October 2004. All samples were stored in glass flasks at 4 °C. Samples of 3 g of seeds from each site were disinfected using the same technique used for roots that was described earlier. The capacity of the disinfected method to eliminate surface microorganisms was evaluated as follows. (1) A 1-g aliquot of disinfected seeds was transferred to 10 ml nutrient broth and incubated with agitation at 120 rpm, 24 h at 30 °C. Then, 1 ml aliquots were sampled and plated for total culturable bacteria on nutrient agar, using the conventional plate count method. Live and dead bacteria (culturable and nonculturable) in the seeds were further verified by two fluorescent techniques described earlier. (2) A second 1 g aliquot of seeds (150 seeds) was placed on the surface of either nutrient agar, tryptic soy agar, or potato dextrose agar. Ten seeds per plate were placed on the surface of the medium (five replicates), all plates were incubated for 24–300 h at 30 ± 2 °C. The area around the germinating seed was checked daily for bacterial and fungal growth.

When no seed surface bacteria were detected in a seed sample, 1 g of disinfected seeds was aseptically homogenized in a mortar with 10 ml PBS. The slurry was centrifuged at 1085 *g* for 10 min and the supernatant (containing bacteria) was collected and seed debris was discarded. The supernatant was then centrifuged at 12,100 *g* for 20 min and the pellet resuspended in 10 ml PBS. Live bacteria was counted using FDA stain and total bacteria, not all necessarily alive, was counted using the FITC stain as described above. Seed inhabiting bacteria were isolated from the seed material using 1 ml homogenized seeds and treated similarly to the procedure of isolation of endophytes from roots on Rennie's medium (Rennie, 1981). Additionally, serial dilutions of the homogenized seeds in PBS were plated (100 μl plate^{-1}) on nutrient agar and tryptic soy agar plates. All plates were incubated at 30 °C for 24–48 h. The isolates obtained were purified in the same media.

At the same time, 150 disinfected seeds were germinated under aseptic conditions in nutrient, tryptic soy, and potato dextrose agar medium at 30 ± 2 °C in the dark during the first 3 days; after that, the seedlings were incubated at 30 ± 2 °C under light (intensity: 70 μmole photon m^{-2} s^{-1}; photo period: 12 h; 10 days). Afterwards, the plantlets were prepared for optical microscopy and scanning electron microscopy (SEM) studies, as described earlier. For bacterial counts, similar to bacterial counts from seeds described earlier, 1 g of seedlings was homogenized in 10 ml PBS.

Table 2
N_2-fixation and phosphate solubilization by endophytic bacteria from cardon seeds and plants.

Plant part	Bacterial isolate	nmole ethylene/culture/h	Phosphate solubilization				Marble (mm)	Limestone (mm)
			Henderson's medium (mm halo)	Pikoskaya's médium (mm halo)				
				Ca	Fe	Al		
Cylinder vascular of root	*Bacillus* sp. ES1	100.40 ± 2.93	ND	ND	ND	ND	ND	ND
	Bacillus sp. ENDO 3	110.40 ± 2.94	ND	16$^{(45)}$	ND	ND	20–28$^{(9)}$	ND
	Bacillus sp. ENDO 4	95.46 ± 3.58	ND	ND	ND	ND	ND	ND
	Bacillus sp. ENDO 5	143.88 + 2.91	ND	18–20 $^{(45)}$	ND	ND	ND	ND
	Bacillus sp. ES2	95.46 ± 3.58	ND	ND	ND	ND	ND	ND
	Unidentified isolates							
	ENDO 2A	111.37 ± 0.67	ND	ND	ND	ND	ND	ND
	ENDO 2B	176.86 ± 3.97	ND	ND	ND	ND	ND	ND
	ENDO 4A	111.81 ± 0.97	ND	13–14 $^{(45)}$	5$^{(45)}$	ND	16–18$^{(9)}$	15–16$^{(9)}$
	ENDO 4C	82.80 ± 0.91	ND	ND	ND	ND	ND	ND
	ENDO 9911		ND	NC	ND	0.5$^{(45)}$	ND	ND
	ENDO 102		ND	ND	ND	ND	20–21$^{(3)}$	23$^{(3)}$
Seed	*Klebsiella* sp. SENDO 1	278.40 ± 5.52	ND	2$^{(16)}$	5$^{(16)}$	ND	ND	ND
	Klebsiella sp. SENDO 2	254.71 ± 2.84	ND	ND	ND	ND	15–17$^{(9)}$	ND
	Bacillus sp. SENDO 6		15–18 $^{(45)}$	13–18$^{(45)}$	ND	ND	22$^{(3)}$	22–23$^{(3)}$
	Staphylococcus sp. SENDO 2		13–15$^{(3)}$	15–17$^{(3)}$	ND	ND	26–28$^{(3)}$	28–29$^{(3)}$
	Pseudomonas sp. SENDO 1		17–20$^{(3)}$	13–15$^{(3)}$	ND	ND	5$^{(3)}$	5$^{(3)}$
	Staphylococcus sp. SENDO 1		10–15$^{(16)}$	15–16$^{(16)}$	ND	ND	23–25$^{(3)}$	26–27$^{(3)}$
	Acinetobacter sp. SENDO 1		16–20$^{(3)}$	15–17$^{(3)}$	ND	ND	29–30$^{(3)}$	26–32$^{(3)}$
	Pseudomonas sp. SENDO 2		10–15$^{(16)}$	15–20$^{(16)}$	2$^{(16)}$	ND	15$^{(3)}$	27–30$^{(3)}$
	Unidentified strains							
	SENDO 1		ND	16–20 $^{(16)}$	ND	ND	ND	ND
	SENDO 4		10–15$^{(16)}$	15–18 $^{(16)}$	ND	ND	23–25$^{(9)}$	25–28$^{(9)}$
	SENDO 412		5$^{(16)}$	5 $^{(16)}$	5$^{(16)}$	ND	ND	ND
	SENDO 119		ND	ND	5$^{(16)}$	ND	ND	ND
	SENDO 2		13–15$^{(3)}$	15–17$^{(3)}$	ND	ND	27$^{(9)}$	25–27$^{(9)}$
	SENDO 513		ND	10$^{(16)}$	ND	ND	ND	ND
	SENDO 311		ND	5$^{(16)}$	ND	ND	ND	ND

Unidentified strains are available from the Environmental Microbiology Group, Center for Biological Research of the Northwest, La Paz, B.C.S., Mexico. Plants are from the ancient lava flow at La Purisima–San Isidro. Numbers in parenthesis indicate when the first solubilization was visually detected (days). Al = $AlPO_4$, Fe = $FePO_4$ $2H_2O$, Ca = $Ca_{10}(OH)_2(PO_4)_6$, the sole phosphorus sources in the medium. ND = No visual solubilization was detected. NC = No definite visible solubilization could be observed. ±Standard deviation.

Possible transfer of bacteria to fruit and seeds was evaluated, as follows: Three mature, closed fruits were surface-disinfected (as described earlier) and cut in half with a sterile scalpel. Fresh samples were prepared for SEM observation. Examination was made from the outside area of the thorns and areolas inward, observing the epicarp, mesocarp, and endocarp tissues and the innermost seed site. Tissue samples and seeds were homogenized in PBS separately, and were plated on nutrient agar for 48 h at 37 °C for total culturable bacterial count in these tissues and for isolation of pure colonial morphotypes.

2.6. Rock sample size and statistical analysis

At least 10 samples were taken from each field site. Each analysis included 8–10 replicates; one piece of root, one Erlenmeyer flask, or one Petri dish served as a replicate. Over 500 SEM images were taken and at least 10 different specimens were taken for particle analysis by the image analyzer. Triplicate samples were analytically assayed. Percentage data was arc sin converted before analysis. One-way ANOVA, followed by Tukey's ad-hoc analysis or Student's *t*-test at $P < 0.05$ was used for statistical analysis with Statistica, version 6 (StatSoft, Tulsa, OK). Numerical data is accompanied by standard errors. The cladogram of the parsimony analysis was obtained using Paup 4.0 software (Sinauer Associates, Sunderland, MA, USA).

3. Results

3.1. Identification of endophytic bacteria colonizing cactus roots growing in soil-free rocks

Endophytic root colonization was usually observed inside cardon roots. After peeling the cell wall of epidermis cells, which was heavily colonized by various rhizoplane bacterial morphotypes, the interiors of the cells were colonized by intracellular endophytes. Later, *in situ* vital staining showed that the bacteria were alive (Puente et al., 2004a; Fig. 4).

From these sampling areas, of the 26 strains of endophytic bacteria, 11 strains were isolated from the vascular system of roots of cardon and 15 strains were isolated from seeds, using two criteria: (1) capacity for nitrogen-fixation *in vitro* and (2) solubilization potential for phosphate, marble, and limestone. Out of these 26 strains, 17 were identified by the 16s rRNA method (Table 2).

3.2. Mineral weathering

To evaluate weathering of igneous rock, six identified endophytic bacteria, together with two plant growth-promoting bacteria (PGPB) known for rock weathering (used as positive controls), and three mixtures of the endophytes with the PGPBs were used. The clearest indication that bacteria could dissolve, hence, weather

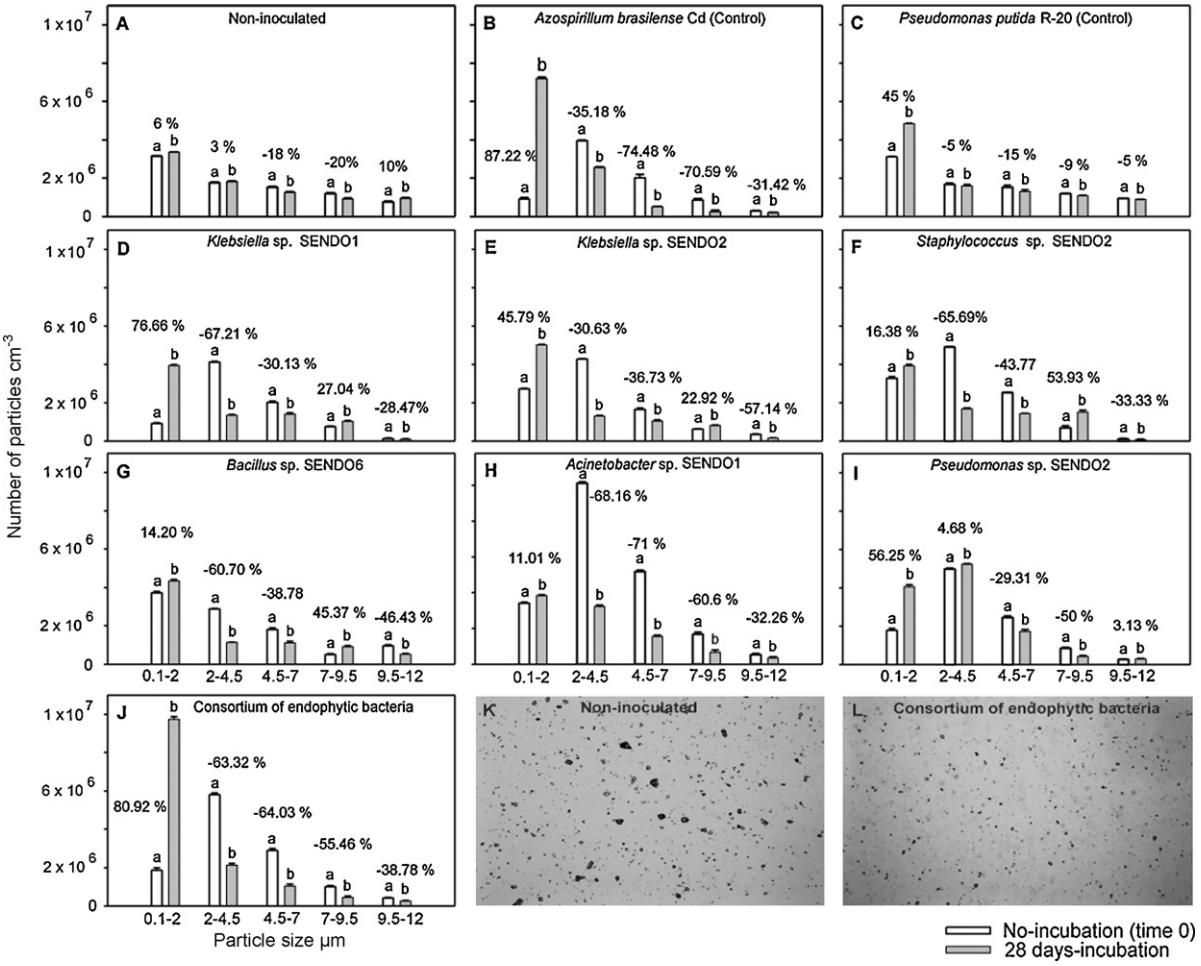

Fig. 1. Reduction in particle size of pulverized extrusive igneous rock after 28 days of incubation with saline (A), *A. brasilense* Cd (control) (B), *Pseudomonas putida* R-20 (control) (C), *Klebsiella* sp. SENDO 1 (D), *K.* sp. SENDO 2 (E), *Staphylococcus* sp. SENDO 2 (F), *Bacillus* sp. SENDO 6 (G), *Acinetobacter* sp. SENDO 1 (H), *Pseudomonas* sp. SENDO 2 (I), Consortium of all endophytic bacteria (J). Micrographs of pulverized rock particles before (K) and after 28 days of incubation (L) using the bacterial consortium listed in J. Columns for each particle size denoted by different letter differ significantly at $P \leq 0.05$ by Student's *t*-test. Values above pairs of column represent the change in the number of particles followed incubation. Bars represent standard error (SE). The absence of SE indicates a negligible value. The experiment was repeated twice.

pulverized rocks was a reduction in particle diameter of powdered grains after incubation (Fig. 1K and L). Although the best weathering agent was the mixture of endophytic bacteria (Fig. 1), each of the endophytes was capable of reducing the size of the particles. Particles >2 μm were reduced; the number of small particles significantly increased (Fig. 1 A–I). Incubation conditions (control flasks without bacteria), produced some weathered particles, but the effects were significantly smaller on reducing size.

Significant reduction in the content of the nine elements assayed occurred with all endophytic bacteria tested, including the mixtures, but to different extents, depending on the species. The maximum reductions in minerals were: K_2O (81.93%), Ca^{2+} (45.81%), Na (91.07%), Fe_2O_3 (30.56%), Mg^{2+} (28.35%), Mn^{2+}

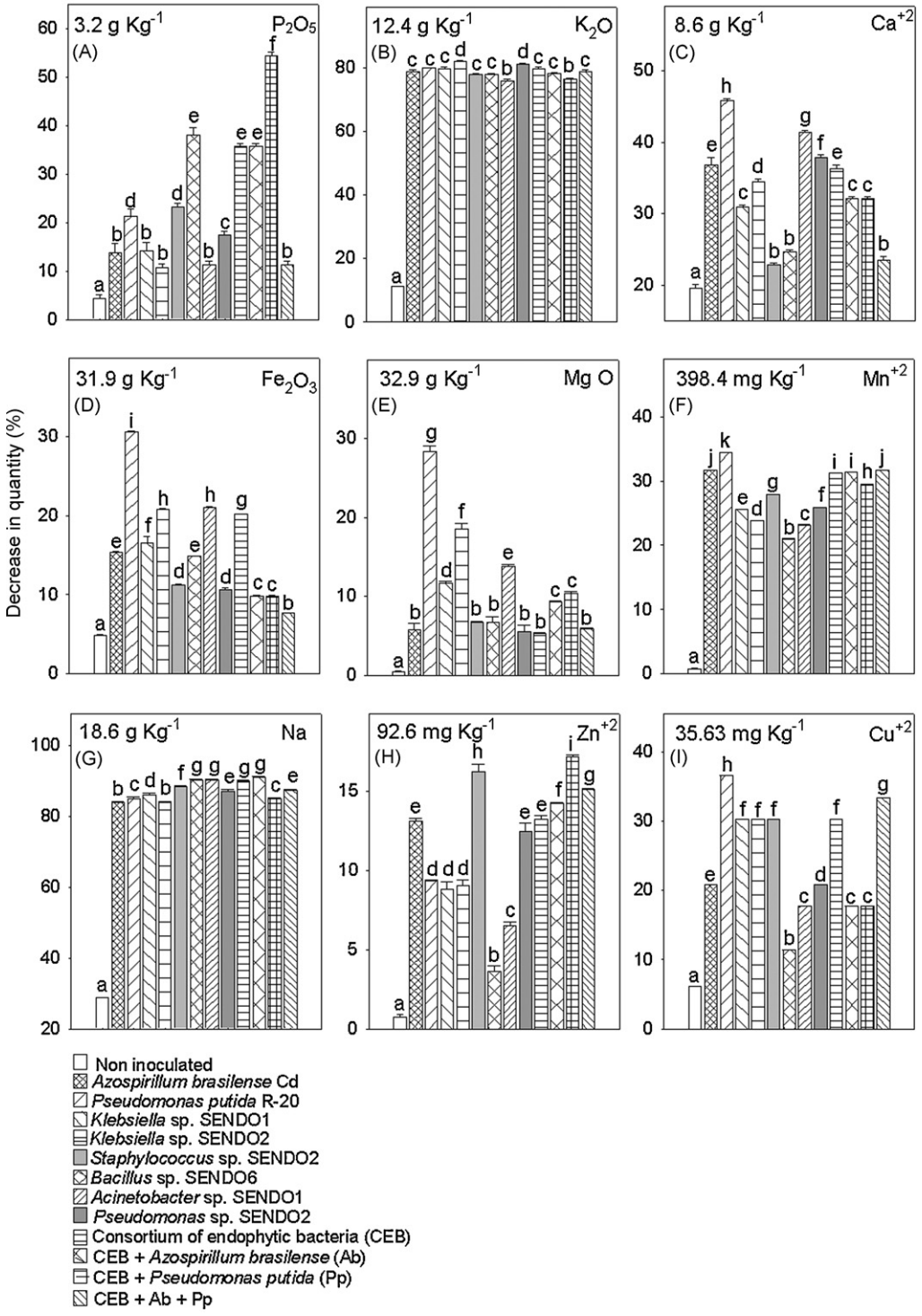

Fig. 2. Analysis of nine compounds in ancient lava: (A) P_2O_5, (B) K_2O, (C) Ca^{2+}, (D) Fe_2O_3, (E) MgO, (F) Mn^{2+}, (G) Na, (H) Zn^{2+}, (I) Cu^{2+} after 28 days of incubation with six endophytic bacterial species, four bacterial consortia, and two control bacteria growing in these rocks. Number in the upper left corner of each subfigure represents the initial quantity of the mineral in the rock. Results are presented as percentage decrease in the element concentration in the rock after incubation with the bacteria. Columns denoted by a different letter in each subfigure differ significantly at $P < 0.05$ by one-way ANOVA. Bars represent standard error (SE). The absence of SE indicates a negligible value. The experiment was repeated twice.

(34.36%), Cu^{2+} (30.27%), P_2O_5 (54.37%), and Zn^{2+} (17.15%) (see Fig. 2).

Over time, all strains released orthophosphate from the pulverized igneous rocks, but *Klebsiella* sp. SENDO 2 and *B.* sp. SENDO 6 released the most orthophosphate, as did the mixtures of endophytic bacteria with the positive controls (Fig. 3A–C). The non-inoculated controls did not release any measurable quantity of orthophosphate.

With *in vitro* assays, the identified strains were evaluated for their ability to dissolve three kinds of insoluble phosphate Ca, Fe, and Al phosphate on two solid media. None was clearly able to dissolve $AlPO_4$. Five strains had limited ability to dissolve $FePO_4{\cdot}2H_2O$, but most strains growing on Pikoskaya's medium dissolved $Ca_{10}(OH)_2(PO_4)_6$ (Table 2). With *in vitro* assays, 12 endophytic strains dissolved pulverized marble and limestone to a great extent (Table 2).

3.3. Physiological characterization of endophytic isolates (temperature and NaCl tolerance, nitrogen-fixation, and organic acid production)

Under natural conditions, air temperature on the igneous rocks regularly exceeds 40 °C in summer. On 7 September 1999 (the hottest month), air temperature was 42 °C and the internal rock temperature was over 60 °C because there is almost no shade and the rock absorbs much radiant energy. The bacteria species growing in solid medium (listed in Tables 1 and 2) were tested for their ability to withstand high temperatures. None grew at 55 °C, but all Gram-positive strains grew well at 45 and 50 °C. Two strains of Gram-negative *Klebsiella* (SENDO 1 and SENDO 2) and *P. putida* SENDO 1 and *Pseudomonas* sp. SENDO 2 did not grow at any of the tested temperatures. When grown in nutrient agar and tryptic soy agar, all bacteria showed tolerance to 3% or more NaCl, one of the main weathered mineral products

After surface sterilization, root samples obtained from the center of the vascular system and the edges of small, adult cardon, yielded potential nitrogen-fixing bacterial populations ranging from $6.40 \times 10^6 \pm 5.27 \times 10^3$ to $0.599 \times 10^6 \pm 1.28 \times 10^3$ CFU g^{-1}, respectively. These bacteria, after enrichment, fixed 154.68 ± 3.23 to 114.30 ± 1.24 nmole ethylene h^{-1} culture^{-1}. With the *in vitro* acetylene reduction assay, 9 endophytic strains isolated from the vascular system and 2 strains from seeds had nitrogen-fixing ability (Table 2). The level of fixed nitrogen varied between endophytic bacteria on the surface of vascular cylinders and bacteria living in the center of vascular cylinders. Nitrogen-fixation and the number of endophytic bacteria from 10 batches of seeds were evaluated. The number of endophytes on Rennie's N-free medium varied from 30 to 450×10^6 CFU ml^{-1} and nitrogen-fixation after enrichment varied from 507.80 to 736.27 nm ethylene h^{-1} culture^{-1}. A linearly significant correlation between the number of endophytes and nitrogen-fixation was found ($Y = 0.61 + 0.29X$; $r = 0.948$; $P < 0.05$). However, only two endophytes (*Klebsiella* sp. SENDO 1 and *Klebsiella* sp. SENDO 2) isolated from seed were nitrogen-fixers from all the morphotypes detected, each fixing 278.40 ± 5.52 and 254.71 ± 2.84 nm ethylene h^{-1} culture^{-1}, respectively.

In liquid culture supplemented with pulverized igneous rock, these six endophytes and the two positive-control PGPB produced 12 volatile and non-volatile organic acids in significant quantities. The most common acid produced by all the strains was gluconic acid, while other acids varied among the strains (Table 3). Additionally, the bacteria species produced unidentified organic acids (5–20) in significant quantities (Table 3, last column).

Table 3
In vitro production of organic acids by some bacterial strains.

Bacteria species	Organic acids (μg ml^{-1})											No. of unidentified organic acids μVa
	Volatile							Non-volatile				
	Gluconic	Propionic	Isovaleric	Heptanoic	Caproic	Acetic	Formic	n-Butyric	Succinic	Lactic	Oxalic	Methylmalonic	
Acinetobacter sp. SENDO1	85992		736					1701			370	61	16 (1507–4.55 × 10^7)*
Bacillus sp. SENDO 6	13992	407	63				258		90295	6			11 (1596–7.42 × 10^6)
Klebsiella sp. SENDO 1	43989		38	195		1931			4915	6020			12 (1520–2.67 × 10^7)
Klebsiella sp. SENDO 2	1291793												12 (1520–2.67 × 10^7)
Staphylococcus sp. SENDO 2	12770						5763				9840		20 (2540–3.58 × 10^7)
Pseudomonas sp. SENDO 2	274634				30						150		20 (1521–5.10 × 10^7)
Positive control													
Pseudomonas putida R-20	14480												5 (1451–2.67 × 10^5)
Azospirillum brasilense Cd	8620	2360	100	250					8150				16 (1626–2.51 × 10^7)

* Range of concentration of unidentified organic acid in parenthesis.

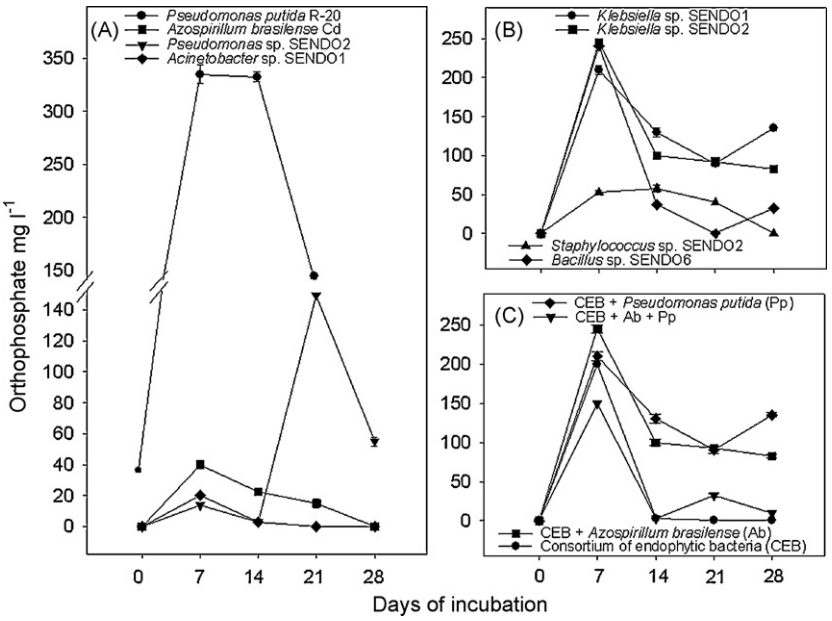

Fig. 3. Solubilization of phosphorus from rocks (release of orthophosphate) with time by endophytic bacteria. *Pseudomonas putida* R-20 and *Azospirillum brasilense* Cd served as control. Bars represent standard error (SE). The absence of SE indicates a negligible value. The experiment was repeated twice.

3.4. Level of endophytes in seeds

Most cross-sections of cardon seeds collected from eight widely separated locations revealed endophytic bacteria in high density under the cortex (Fig. 4A) All eight seed batches contained high number of endophytes and most proved to be viable using fluorescent vital staining (FDA) (Fig. 4B).

Four independent counting methods (fluorescent staining and plate count methods) on two batches of cardon seeds from widely separated areas (San Jose del Cabo and El Rosario) revealed numerous bacteria residing on the surface of seeds. FITC count revealed $19.6 \times 10^6 \pm 1.83 \times 10^6$–$20.5 \times 10^6 \pm 1.37 \times 10^6$ CFU g^{-1} seeds for the two locations, respectively, where at least $15.2 \times 10^6 \pm 0.547 \times 10^6$ CFU g^{-1} seeds were alive, according to FDA vital staining. The plate count methods revealed natural populations greater than 10^9 CFU g^{-1} seeds. Seeds whose surface was disinfected revealed no bacteria in plate count and FDA methods and only 4.76 ± 0.54–5.07 ± 0.07 by FITC, presumably dead cells counted by the fluorescent dye.

Enumeration of the endophytic culturable populations by the four methods revealed that populations derived from the two locations (the junction of the San Jose and El Rosario roads and from La Purisima–San Isidro), fluctuated among the detection methods. For FDA, $4.55 \times 10^6 \pm 0.00403 \times 10^6$–$4.60 \times 10^6 \pm 0.00135 \times 10^6$; for FITC, $9.15 \times 10^6 \pm 0.00672 \times 10^6$–$9.21 \times 10^6 \pm 0.00672 \times 10^6$; for nutrient agar, $3.55 \times 10^6 \pm 0.00270 \times 10^6$–$4.40 \times 10^6 \pm 0.00266 \times 10^6$; and for tryptic soy agar, $4.64 \times 10^6 \pm 0.00648 \times 10^6$–$5.16 \times 10^6 \pm 0.00113 \times 10^6$, respectively, for the two locations. Evaluation of eight other seeds batches from different locations where cardon were growing revealed similar-sized endophytic populations (Fig. 4C). Every seed batch collected from wild plants had an endophytic population.

3.5. Possible transfer of endophytic bacteria from seed to shoot and from fruit to seed

Seeds with a disinfected surface were suspended in liquid nutrient broth or germinated on nutrient agar, did not have bacteria residing on the surface of the seeds later in the assay. Untreated natural seeds created large populations of bacteria in both media after overnight incubation. Crushing disinfected seeds, followed by vital staining with two different fluorescent dyes, revealed many live endophytic bacteria, as described above. When these seeds germinated, histological cuttings from the root system and shoot of the seedlings revealed endophytic bacteria, mainly in the intercellular spaces of the root cortex and vascular system (Fig. 4D and E). This was corroborated by SEM observations of the same seedlings cut longitudinally (Fig. 4F). Possible transfer of endophytes from mature fruit to seeds was observed upon opening intact fruit from collection sites. The interior of surface-disinfected fruit was heavily colonized with endophytic bacteria. Fifteen different culturable morphotypes were isolated, but were not identified. Bacteria were found in the endocarp, mesocarp, and epicarp, on the surface, and at the embryonic site of seeds (Fig. 5).

3.6. Comparisons of endophytic strains obtained from seeds and roots of cardon cacti with rhizoplane populations

Partial sequencing of the 16S rRNA gene of the culturable bacterial strains indicated that, although the genera of these strains can be determined, they have lower than the required 98.7% similarity to known species in the GenBank repository. Therefore, they may be unknown species. However, evaluation of genetic similarities based on rRNA sequences among 25 morphotypes obtained from seeds, interior of roots, and rhizoplane populations indicate high similarity. Pairs of some endophytes and rhizoplane bacteria, such as *Bacillus* sp., *Klebsiella* sp., *and Staphylococcus* sp. had up to 100% similarity in their 16S rRNA gene. Each of the two main clusters that were detected had isolates in the three habitats (Fig. 6).

4. Discussion

Soil formation in extremely hot deserts is a slow process, particularly where only the physical and chemical climatic processes are involved. Biological processes might accelerate soil formation by plants colonizing bare rocks. In most deserts, higher plants seldom colonize rocks, leaving the biological process to microorganisms. Sometimes, as found in the desert highlands of the Baja California Peninsula, there is massive colonization of bare rocks and cliffs by several plant species that visibly and significantly

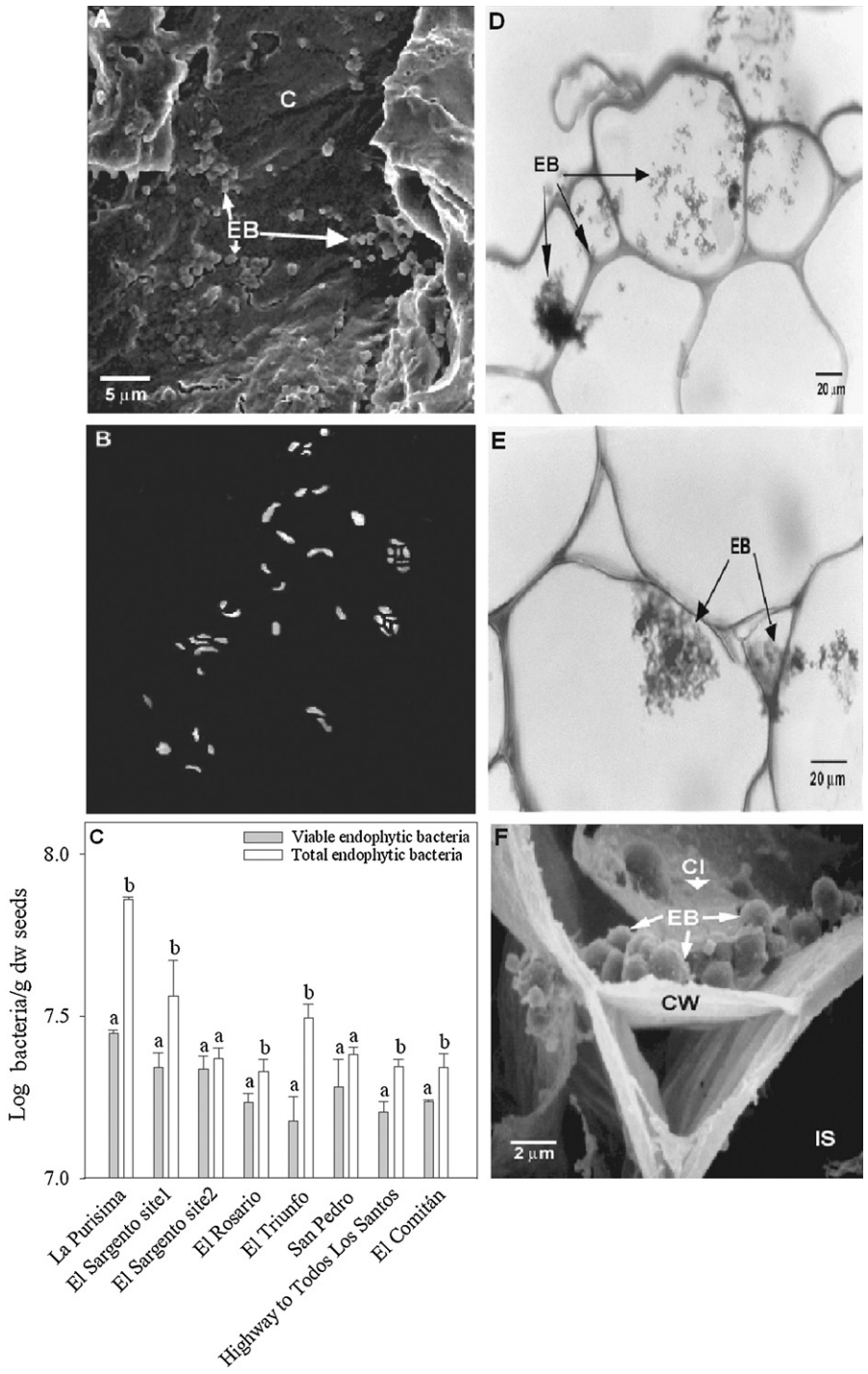

Fig. 4. (A) Typical cross-section of a seed of cardon collected from wild tree in El Comitan federal natural preserve. Endophytic bacteria are located under the cortex (arrows). (B) Fluorescent vital staining of these seeds (FDA). EB-endophytic bacteria. (C) Viable (FDA) and total (FITC) endophytic bacteria in seeds of cardon cacti *Pachycereus pringlei* collected in different locations in the state of Baja California Sur, Mexico. Pairs of columns denoted by a different letters differ significantly at $P \leq 0.05$ by Student's *t*-test. Bars represent standard error. (D) Light micrographs of cross-sections of the cortex area of the root and shoot (E) of naturally grown cardon seedling. (F) SEM micrograph of a cross-section of the transition section between root and shoot of germinating cardon seedling containing endophytic bacteria. CI = cell interior; CW = cell wall; EB = endophytic bacteria; IS = intercellular spaces.

degrade rock, accumulating isolated patches of down slope soil that support diverse plant species (Bashan et al., 2002, 2006). Our working hypothesis was that root-colonizing microorganisms (rhizoplane and endophytic) in these plants directly participate in rock weathering and perhaps supply plants with released inorganic nutrients and nitrogen through nitrogen-fixation. This effect on plant development accelerates soil formation in otherwise barren landscapes.

Evaluation of rhizoplane bacteria populations on roots growing "aeroponically" on rocks in the absence of soil revealed large numbers of bacteria and fungi capable of dissolving minerals; some are nitrogen-fixers (Puente et al., 2004a). This study demonstrated that cardon roots contained also endophytic bacteria that are capable of fixing nitrogen, dissolving several essential minerals, and changing insoluble to soluble phosphates. These traits characterize both populations as plant growth-promoting bacteria of wild plants in arid

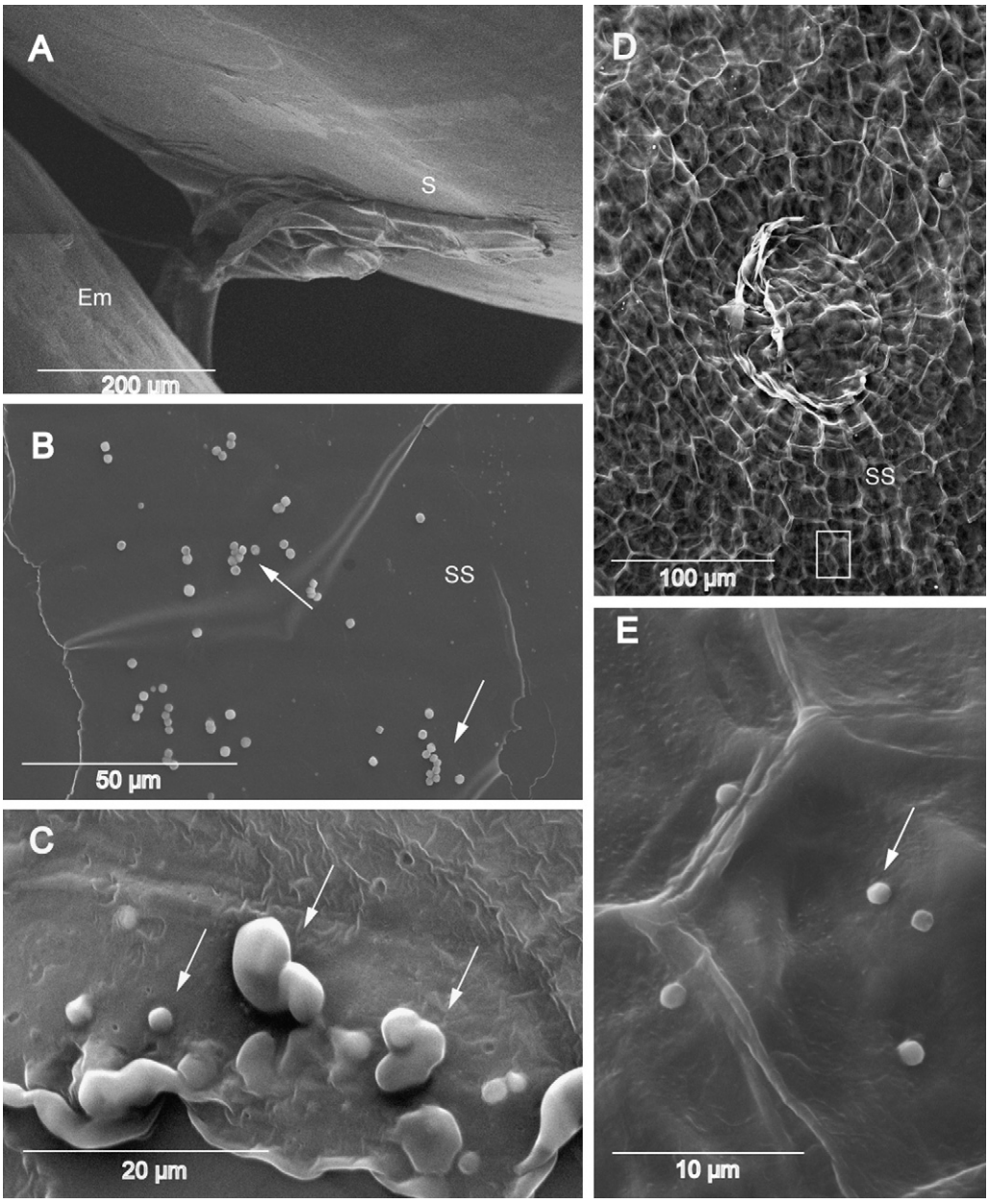

Fig. 5. Endophytes inside mature fruit and seeds of cardon. (A) seed inside the fruit connected to the endocarpe, (B,C) Higher magnification of seed surface (arrows indicate bacteria), (D) Detached (mechanically) seed from the endocarpe, (E,) Higher magnification of the connecting tissue marked in D, (Arrows indicate endophytic bacteria). Abbreviations: S-seed; SS-seed surface; En-endocarp.

lands, resembling the PGPB phenomenon in agriculture (Kobayashi and Palumbo, 2000; Bashan and de-Bashan, 2005).

In deserts, nitrogen-fixing bacteria are uncommon in rocks without vegetation. Nitrogen for endolithic microorganisms in deserts is fixed by lightning or auroras, and is then deposited in rock cavities by occasional precipitation (Friedmann and Kibler, 1980). Diazotrophic PGPB of the genus *Azospirillum* promotes establishment and growth of cacti in eroded soils and contributes nitrogen to the plants (Puente and Bashan, 1993; Bashan et al., 1999; Carrillo-Garcia et al., 2000). Diazotrophic, endophytic *Pseudomonas stutzeri* was found in the desert epiphyte *Tillandsia recurvata* (Puente and Bashan, 1994) that is common on the western coastal plains of the Baja California Peninsula of Mexico. This suggests a possible role for diazotrophs in nitrogen nutrition of desert plants. In this study, the high levels of nitrogen-fixation, although assessed *in vitro*, and associated with weathering of igneous rock, are probably the result of microbial activity in the roots of cacti. Although unproven directly, it is likely that part of the fixed nitrogen is transferred to the plants, since experimental plants inoculated with diazotrophic endophytes showed no sign of nitrogen deficiency after one year of cultivation, even with bacteria exhibiting only low nitrogen-fixation capacity with *in vitro* assays and the rock on which they live does not contain any detectable nitrogen (Bashan et al., 2002).

The accelerated breakdown of rock by plants, in contrast to common weathering, can be partly attributed to the solubilizing activity of microorganisms that colonize plant roots (Gyaneshwar et al., 1998), organic acids exuded by the roots (Lynch and Whipps, 1990), and microorganisms (this study). Cardon cacti inoculated with the PGPB *A. brasilense* excreted more protons and organic acids, which lowered the rhizosphere pH and made phosphorus more available to the cardon (Carrillo et al., 2002; Rodriguez et al., 2004). Rhizosphere microflora (endo- and ectomycorhiza and rhizobacteria) of maize, rice, and pine trees promote the transformation of minerals (Berthelin et al., 1991). Our study showed that endophytes in cacti

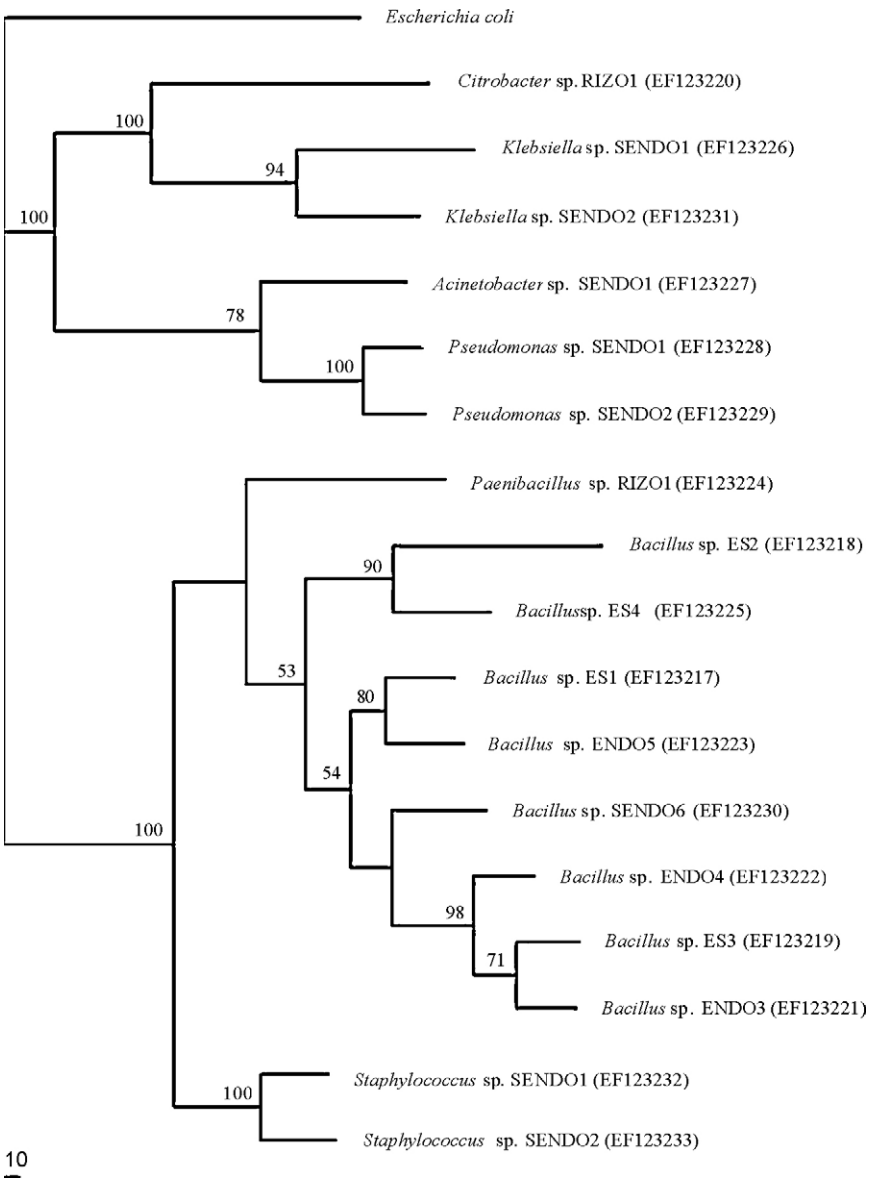

Fig. 6. A phylogenetic tree developed by parsimony analysis of the strains used in this study. Partial sequences (995 pb) of 16S rRNA of bacteria from seeds, rhizoplane, and endophytes of cardon cacti were used. *E. coli* was used as an external control. The bar indicates the number of bases that differ between sequences. Numbers on each node indicate the frequency of the clusters (in percent) obtained according to the bootstrap analysis, using 5000 replicates. In parentheses appear the access numbers of the sequence in the GenBank registry. Consistency Index was 0.63; retention index was 0.606; and the Homoplasticity Index was 0.37.

can grow on relatively insoluble phosphate powder and transform it to plant-available orthophosphate. Most of the endophytic bacteria that were isolated dissolved calcium phosphate, which is easier to dissolve than Al and Fe phosphates (Illmer and Schinner, 1995; Illmer et al., 1995), which are found in igneous rocks (Bashan et al., 2002). Several Antarctic lichens weather andesitic basalt, similar to the rock evaluated in this study, and transforming the original minerals (Ascaso et al., 1990). The data in this study supports our previous results (Bashan et al., 2002; Puente et al., 2004a) that showed that the breakdown of rock minerals and changes in mineral composition in rock cavities colonized by pioneer desert plants is an active process.

This study, together with the second part (Puente et al., 2009), provides some evidence supporting a process where bacteria may be transferred throughout the cycle of growth and contribute to rock weathering, soil formation, and promotion of vegetative growth in desert areas. This model, still lacking some experimental evidence, implies that the source of these microorganisms is the endophytic bacteria in seeds. Cacti form an association with these microorganisms, and as prolific endophytes, they readily transfer to the shoots, fruit, and seeds. The evolutionary advantage for this association with endophytes is that it supports establishment and growth of young cacti on barren rocks and does not need to acquire the bacteria from the environment.

An attempt to identify the species level of these bacterial strains indicates that they are apparently unknown species within known genera; several of the endophytes and rhizoplane bacteria are very closely related strains. Although some of the bacteria populations in seeds and the rhizoplane are different strains, the multitude of strains detected, and mostly unidentified, make it probable, but not fully confirmed, that strains appear in both the rhizoplane and in the seeds, but in different proportions during plant development. Similarities between isolates from tree roots, healthy crops, and weeds obtained from different locations suggest that these are facultative endophytes and are capable of living outside plant tissues as rhizosphere bacteria (Di Fiori and Del Gallo, 1995; Lodewyckx et al., 2002). Additionally, based on identification of strains isolated from different parts of roots, it appeared that many endophytic bac-

teria taxa from sweet corn or cotton are also common soil bacteria (McInroy and Kloepper, 1994, 1995; J. McInroy, pers. comm., 2008).

In summary, this study demonstrated that populations of culturable endophytic bacteria, comparable in size to endophytic populations of some crop plants (Kobayashi and Palumbo, 2000), occur on cactus roots growing in rocks where soil is absent. These microbes, together with the roots they colonize, might significantly increase weathering of igneous rocks in a hot desert. Cactus seeds contain rock-weathering endophytic bacteria that allows seedlings to establish themselves on very hot, barren rocks; hence, do not need to acquire the bacteria from the environment. In young seedlings, the endophytes may migrate from the seed to the roots and to the rhizoplane, where, in the process of development in the rhizoplane, they dissolve the rock minerals and migrate throughout the interior of the growing seedling. This newly discovered plant–bacteria symbiosis is a biological factor in accelerating soil formation in rocky deserts containing little or no soil. This model, only partially validated by the data presented, shows that cacti gains an evolutionary advantage by acquiring a diverse population of endophytes that allows this plant to gain a foothold on highly uncompromising terrain.

Acknowledgements

We thank Al Soeldner (Oregon State University, Corvallis, Oregon) and Vladimir Lebsky, Maria del Carmen Rodríguez, and Ariel Cruz (CIBNOR) for assisting in histology, image analysis, and electron microscopy, and the following staff at CIBNOR: Baudilio Acosta and Griselda Peña Armenta for mineral analysis, Manuel Trasviña for phosphorus analysis, and Sonia Rocha and Dolores Rondero for nitrogen analysis. The16S rRNA gene sequence analysis was done as a service by the Department of Crop and Soil Sciences, Oregon State University, Corvallis, Oregon. Yoav Bashan participated in this study in the memory of the late Avner Bashan of Israel. This work was partially supported by Consejo Nacional de Ciencia y Tecnologia of Mexico (CONACYT, project 50052-Z), the Pacific Northwest Research Station of the USDA Forest Service, Corvallis, Oregon, USA, and the Bashan Foundation of Oregon, USA.

References

Adams, J.B., Palmer, F., Staley, J.T., 1992. Rock weathering in deserts: mobilization and concentration of ferric iron by microorganisms. Geomicrobiol. J. 10, 99–114.
Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.L., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
Ascaso, C., Sancho, L.G., Rodriguez-Pascual, C., 1990. The weathering action of saxicolous lichens in maritime Antarctica. Polar Biol. 11, 33–40.
Babiuk, L.A., Paul, E.A., 1970. The use of fluorescein isothiocyanate in the determination of the bacterial biomass of grassland soil. Can. J. Microbiol. 16, 57–62.
Bach, W., Edwards, K.J., 2003. Iron and sulfide oxidation within the basaltic ocean crust: implications for chemolithoautotrophic microbial biomass production. Geochim. Cosmochim. Acta 67, 3871–3887.
Barker, W.W., Banfield, J.F., 1998. Zones of chemical and physical interaction at interfaces between microbial communities and minerals: a model. Geomicrobiol. J. 15, 223–244.
Bashan, Y., de-Bashan, L.E., 2005. Bacteria/plant growth-promotion. In: Hillel, D. (Ed.), Encyclopedia of Soils in the Environment, vol. 1. Elsevier, Oxford, UK.
Bashan, Y., Li, C.Y., Lebsky, V.K., Moreno, M., de-Bashan, L.E., 2002. Primary colonization of volcanic rocks by plants in arid Baja California, Mexico. Plant Biol. 4, 392–402.
Bashan, Y., Rojas, A., Puente, M.E., 1999. Improved establishment and development of three cacti species inoculated with *Azospirillum brasilense* transplanted into disturbed urban desert soil. Can. J. Microbiol. 45, 441–451.
Bashan, Y., Vierheilig, H., Salazar, B.G., de-Bashan, L.E., 2006. Primary colonization and breakdown of igneous rocks by endemic, succulent elephant trees (*Pachycormus discolor*) of the deserts in Baja California, Mexico. Naturwissenschaften 93, 344–347.
Bawden, T.M., Einaudi, M.T., Bostick, B.C., Meibom, A., Wooden, J., Norby, J.W., Orobona, M.J.T., Chamberlain, C.P., 2003. Extreme ^{34}S depletions in ZnS at the Mike gold deposit, Carlin Trend Nevada: evidence for bacteriogenic supergene sphalerite. Geology 31, 913–916.
Berthelin, J., Leyval, C., Laheurte, F., de Giudici, P., 1991. Involvement of roots and rhizosphere microflora in the chemical weathering of soil minerals. In: Atkinson, D. (Ed.), Plant Root Growth: An Ecological Perspective. Blackwell Scientific Publications, Oxford, U.K.
Carrillo, A.E., Li, C.Y., Bashan, Y., 2002. Increased acidification in the rhizosphere of cactus seedlings induced by *Azospirillum brasilense*. Naturwissenschaften 89, 428–432.
Carrillo-Garcia, A., Bashan, Y., Bethlenfalvay, G.J., 2000. Resource-island soils and the survival of the giant cactus cardon of Baja California Sur. Plant Soil 218, 207–214.
Chang, T.T., Li, C.Y., 1998. Weathering of limestone, marble, and calcium phosphate by ectomycorrhizal fungi and associated microorganisms. Taiwan J. Forest Sci. 13, 85–90.
De la Torre, M.A., Gomez-Alarcon, G., Palacios, J.M., 1993. In vitro biofilm formation by *Penicillium frequentans* strain on sandstone, granite, and limestone. App. Microbiol. Biotechnol. 40, 408–415.
Di Fiori, S., Del Gallo, M., 1995. Endophytic bacteria: their possible role in the host plant. In: Fendrik, I., Del Gallo, M., Vanderleyden, J., de Zamaroczy, M. (Eds.), *Azospirillum* VI and Related Microorganisms. Springer-Verlag, Berlin.
Edwards, K.J., Rogers, D.R., Wirsen, C.O., McCollom, T.M., 2003. Isolation and characterization of novel psychrophilic, neutrophilic, Fe-oxidizing, chemolithoautotrophic alpha and gamma Proteobacteria from the deep sea. Appl. Environ. Microbiol. 69, 2906–2913.
Feder, N., O'Brien, T.P., 1968. Plant microtechnique: some principles and new methods. Am. J. Bot. 55, 123–142.
Ferris, F.G., Lowson, E.A., 1997. Ultrastructure and geochemistry of endolithic microorganisms in limestone of the Niagara Escarpment. Can. J. Microbiol. 43, 211–219.
Friedmann, E.I., Kibler, A.P., 1980. Nitrogen economy of endolithic microbial communities in hot and cold deserts. Microb. Ecol. 6, 95–100.
Gorbushina, A.A., Krumbein, W.E., Volkmann, M., 2002. Rock surfaces as life indicators: new ways to demonstrate life and traces of former life. Astrobiology 2, 203–213.
Goudie, A.S., Parker, A.G., 1999. Experimental simulation of rapid rock block disintegration by sodium chloride in a foggy coastal desert. J. Arid Environ. 40, 347–355.
Gyaneshwar, P., Kumar, G.N., Parekh, L.J., 1998. Effect of buffering on the phosphate-solubilizing ability of microorganism. World J. Microbiol. Biotechnol. 14, 669–673.
Henderson, M.E.K., Duff, R.B., 1963. The release of metallic and silicate ions from minerals, rocks, and soils by fungal activity. J. Soil Sci. 14, 236–246.
Hinsinger, P., Gilkes, R.J., 1993. Root-induced irreversible transformation of trioctahedral mica on the rhizosphere of rape. J. Soil Sci. 44, 535–545.
Hinsinger, P., Gilkes, R.J., 1995. Root-induced dissolution of phosphate rock in the rhizosphere of lupines grown in alkaline soil. Aust. J. Soil Res. 33, 477–489.
Hirsch, P., Eckhardt, F.E.W., Palmer Jr., R.J., 1995a. Methods for the study of rock-inhabiting microorganisms—a mini review. J. Microbiol. Methods 23, 143–167.
Hirsch, P., Eckhardt, F.E.W., Palmer Jr., R.J., 1995b. Fungi active in weathering of rock and stone monuments. Can. J. Bot. 73 (Suppl. 1), 1384–1390.
Holguin, G., Guzman, M.A., Bashan, Y., 1992. Two new nitrogen-fixing bacteria from the rhizosphere of mangroves trees, isolation, identification and in vitro interaction with rhizosphere *Staphylococcus* sp. FEMS Microbiol. Ecol. 101, 207–216.
Hossner, L.R., Doolittle, J.J., 2003. Iron sulfide oxidation as influenced by calcium carbonate application. J. Environ. Qual. 32, 773–780.
Illmer, P., Schinner, F., 1995. Solubilization of inorganic calcium phosphate-solubilization mechanisms. Soil Biol. Biochem. 27, 257–263.
Illmer, P., Barbato, A., Schinner, F., 1995. Solubilization of hardy-soluble $AlPO_4$ with P-solubilizing microorganisms. Soil Biol. Biochem. 27, 265–270.
Ingham, E.R., Klein, D.A., 1984. Soil fungi: Relationships between hyphal activity and attaining with fluorescein diacetate. Soil Biol. Biochem. 16, 273–278.
Jackson, M.L., 1958. Soil Chemical Analysis. Prentice-Hall, Englewood Cliffs, NJ.
Kingston, H.M., 1994. EPA Method 3015 Microwave Assisted Acid Digestion of Aqueous Samples and Extracts. Dusquesne University, Pittsburgh, PA.
Kobayashi, D.Y., Palumbo, J.D., 2000. Bacterial endophytes and their effects on plants and uses in agriculture. In: Bacon, C.W., Whites Jr., J.F. (Eds.), Microbial Endophytes. Marcel Decker, New York.
Lynch, J.M., Whipps, J.M., 1990. Substrate flow in the rhizosphere. Plant Soil 129, 1–10.
Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S., Mezgeay, M., van der Lelie, D., 2002. Endophytic bacteria and their potential applications. Crit. Rev. Plant Sci. 21, 583–606.
McInroy, J.A., Kloepper, J.W., 1994. Novel bacterial taxa inhabiting internal tissue of sweet corn and cotton. In: Ryder, M.H., Stephens, P.M., Bowen, G.D. (Eds.), Improving Plant Productivity with Rhizosphere Bacteria. CSIRO, Melbourne, Australia.
McInroy, J.A., Kloepper, J.W., 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil 173, 337–342.
Meyer, J.R., Linderman, R.G., 1986. Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth-promoting bacterium, Pseudomonas putida. Soil Biol. Biochem. 18, 185–190.
Palmer Jr., R.J., Siebert, J., Hirsch, P., 1991. Biomass and organic acids in sandstone of a weathering building production by bacterial and fungal isolates. Microb. Ecol. 21, 253–266.
Pikoskaya, R.I., 1948. Mobilization of phosphates in soil in relation with vital activity of some microbial species. Mikrobiologiya 17, 362–370 (in Russian).

Puente, M.E., Bashan, Y., 1993. Effect of inoculation with *Azospirillum brasilense* strains on the germination and seedlings growth of the giant columnar cardon cactus (*Pachycereus pringlei*). Symbiosis 15, 49–60.

Puente, M.E., Bashan, Y., 1994. The desert epiphyte *Tillandsia recurvata* harbors the nitrogen-fixing bacterium *Pseudomonas stutzeri*. Can. J. Bot. 72, 406–408.

Puente, M.E., Bashan, Y., Li, C.Y., Lebsky, V.K., 2004a. Microbial populations and activities in the rhizoplane of rock-weathering desert plants I. Root colonization and weathering of igneous rocks. Plant Biol. 6, 629–642.

Puente, M.E., Li, C.Y., Bashan, Y., 2004b. Microbial populations and activities in the rhizoplane of rock-weathering desert plants II. Growth promotion of cactus seedlings. Plant Biol. 6, 643–650.

Puente, M.E., Li, C.Y., Bashan, Y. 2009. Endophytic bacteria in cacti seeds can improve the development of cactus seedlings. Environ. Exp. Bot. doi:10.1016/j.envexpbot.2009.04.007.

Rennie, R.J., 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. 27, 8–14.

Rodriguez, H., Gonzalez, T., Goire, I., Bashan, Y., 2004. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. Naturwissenschaften 91, 552–555.

Strzelczyk, E., Li, C.Y., 2000. Bacterial endobionts in the big non-mycorrhizal roots of Scots pine (*Pinus sylvestris* L). Microbiol. Res. 155, 229–232.

Sun, H.J., Friedmann, E.I., 1999. Growth on geological time scales in the Antarctic cryptoendolithic microbial community. Geomicrobiol. J. 16, 193–202.

Vazquez, P., Holguin, G., Puente, M.E., Lopez-Cortes, A., Bashan, Y., 2000. Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves growing in a semiarid coastal lagoon. Biol. Fertil. Soils 30, 460–468.

Yamanaka, T., Li, C.Y., Bormann, B., Okabe, H., 2003. Tripartite associations in an alder: effects of *Frankia* and *Alpova diplophloeus* on the growth, nitrogen fixation and mineral acquisition of *Alnus tenuifolia*. Plant Soil 254, 179–186.